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(54) Title: OPTICAL IMAGING CONTRAST AGENTS

(57) Abstract: The invention provides contrast agents for optical imaging of oesophageal cancer and Barrett's oesophagus in patients. The contrast agents may be used in diagnosis of oesophageal cancer and Barrett's oesophagus, for follow up of progress in disease development, for follow up of treatment of oesophageal cancer and Barrett's oesophagus and for surgical guidance. Further, the invention provides methods for optical imaging of oesophageal cancer and Barrett's oesophagus in patients.

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# Optical imaging contrast agents

## Field of the invention

The present invention provides contrast agents for optical imaging of oesophageal cancer and Barrett's oesophagus in patients. The contrast agents may be used in diagnosis of oesophageal cancer and Barrett's oesophageus, for follow up of progress in disease development, and for follow up of treatment of oesophageal cancer and Barrett's oesophagus.

The present invention also provides new methods of optical imaging of oesophageal cancer and Barrett's oesophageus in patients, for diagnosis and for follow up of disease development and treatment of oesophageal cancer and Barrett's oesophagus.

## Description of related art

Oesophageal cancer is not among the most frequent forms of cancer and less than 5% of all reported cancer cases are oesophageal cancer. However, 30 000 new cases are diagnosed per year in USA. Oesophageal cancer is predominantly a disease of the male. The occurrence of the disease varies from country to country with high occurrence in for example India, Japan, Russia, China, United Kingdom and Middle East.

The main risk factors for oesophageal cancer include tobacco, alcohol and the diet. Oesophageal cancer is divided into two major types, squamous cell carcinoma and adenocarcinoma, depending on the type of cells that are malignant. Barrett's oesophagus is a premalignant condition and the presence is associated with increased risk for development of oesophageal cancer; especially adenocarcinoma. Chronic reflux increases risk for Barrett's oesophagus, and it has therefore been suggested that gastro oesophageal reflux (GERD) is a risk factor for oesophageal cancer.

Adenocarcinoma of the oesophagus is more prevalent than squamous cell carcinoma in US and Western Europe.

Oesophageal cancer can be a treatable disease but is rarely curable. The overall 5-year survival rate is between 5% and 30%. Data from US show a 5-year survival rate of about 5%. Early diagnosis of oesophageal cancer improves the survival rate of the

patient. Primary treatment includes surgery alone or chemotherapy in combination with radiation. Chemotherapy used in treatment of oesophageal cancer includes 5-fluorouracil and cisplatin. Lack of precise pre-operative staging is a major clinical problem.

US 6,035,229 (Washington Research Foundation) describes a system for detecting Barrett's oesophagus utilizing an illumination and imaging probe at the end of a catheter. The document does not disclose any optical contrast agent.

US 5,888,743 (Das) describes an in vitro method for the diagnosis of benign Barrett's epithelium and Barrett's derived adenocarcinoma comprising a monoclonal antibody that reacts with cells.

US 4,243,652 (The Procter & Gamble Company) describes a gastrointestinal scanning agent also to be used for visualization of the oesophageal entry. The agent comprises a gamma radiation emitting radionuclide.

Oesophageal cancer and Barrett's oesophagus are still a challenge to diagnose and treat. There is a need for improved diagnostic methods, especially for diagnosis of oesophageal cancer and Barrett's oesophagus in an early stage with good reliability. Surprisingly, we have discovered that the use of optical imaging methods and new contrast agents fulfil these requirements.

**Summary of the invention**

The present invention provides an optical imaging contrast agent with affinity for an abnormally expressed biological target associated with oesophageal cancer and Barrett's oesophagus.

The invention is also described in the claims.

The following definitions will be used throughout the document:

Oesophageal cancerous tissue: The condition includes alterations in the oesophageal tissue wherein the two major types are squamous cell carcinoma and adenocarcinoma. This also includes oesophageal tissue that shows metaplastic alterations characteristic for Barrett's oesophagus, such as areas of columnar instead of squamous epithelium. Metaplastic oesophageal tissue in general, particularly

tissue that shows progression towards malignancy, involving larger parts of the oesophagus and including invasion of adjacent tissue are also included. Metastases from oesophageal carcinoma are also considered as oesophageal cancerous tissue.

Abnormally expressed target: A target that is either overexpressed or downregulated in oesophageal cancerous tissue.

Overexpressed target: A receptor, an enzyme or another molecule or chemical entity that is present in a higher amount in oesophageal cancerous tissue than in normal tissue.

Downregulated target: A receptor, an enzyme or another molecule or chemical entity that is present in a lower amount in oesophageal cancerous tissue than in normal tissue.

**Detailed description of the invention**

A first aspect of the present invention is an optical imaging contrast agent for imaging of oesophageal cancer and Barrett's oesophagus. By the term optical imaging contrast agent, or just contrast agent, we mean a molecular moiety used for enhancement of image contrast *in vivo* comprising at least one moiety that interacts with light in the ultraviolet, visible or near-infrared part of the electromagnetic spectrum.

The contrast agent has affinity for an abnormally expressed target associated with oesophageal cancer or Barrett's oesophageus.

Oesophageal cancerous tissue containing a downregulated target is identified by a low amount of bound imaging agent compared to normal tissue. In this situation, the amount of imaging agent should be less than 50 % of that in normal tissue, preferably less than 10 %.

Preferred contrast agents according to the invention, have affinity for an overexpressed target associated with oesophageal cancer or Barrett's oesophagus. Preferred targets are those targets that are more than 50 % more abundant in oesophageal cancerous tissue than in surrounding tissue. More preferred targets are those targets that are more than two times more abundant in oesophageal cancerous tissue than in surrounding tissue. The most preferred targets are those targets that

are more than 5 times more abundant in oesophageal cancerous tissue than in surrounding tissue.

In a further aspect of the invention, targets that are mutated in oesophageal cancerous tissue can be identified by lack of binding of an imaging agent that does bind to normal tissue; alternatively, the imaging agent might be directed specifically towards the mutated target, and binding to normal tissue would be minimal. The mutated target can be a protein in oesophageal cancerous tissue that is altered as a result of a germline or somatic mutation, and including alterations resulting from differential splicing of RNA and changes in post-translational modifications, particularly glycosylation patterns, but not limited to these types of alterations.

Relevant groups of targets are receptors, enzymes, nucleic acids, proteins, lipids, other macromolecules as, for example, lipoproteins and glycoproteins. The targets may be located in the vascular system, in the extracellular space, associated with cell membranes or located intracellularly.

Preferred groups of targets are antigens, proteins involved in cell cycle regulation or intracellular signalling, enzymes, hormones, growth factors, cytokines and similar proteins and peptides, cytokeratins, cell-surface receptors associated with Barrett's oesophagus or oesophageal cancer.

The following biological targets are preferred targets for contrast agents for optical imaging of oesophageal cancer and Barrett's oesophagus:

**Targets that are overexpressed in Barrett's oesophagus:**

Antigens:

MUC5AC, MUC3, MUC2, MUC6, MUC2, CD34, PCNA, MUC2, Sulfo-Lewis(a).

Proteins involved in cell cycle regulation or intracellular signalling:

PCNA, enzymes of polyamine metabolism, p53, p63, KI67, p53, c-ras, c-src, ß-catenin, Mcm2, Mcm5.

Hormones, growth factors, cytokines and similar proteins and peptides:

VEGFs, IL1ß, IL-8, IL-10, TGF-α, EGF, TGF-α, TNF-α.

Cytokeratins etc.:

CK7, CK20, CK 8, CK 13, CK 18, CK 19.

Cell-surface receptors:
Epidermal growth factor receptor (EGFR), c-erb2, CD44H, CD44V6, c-myc, Guanylyl cyclase.

Others:
c-jun, E-cadherin, ß-galactosidase, metallothionein, telomerase.

More preferred targets that are overexpressed in Barrett's oesophagus are E-cadherin, guanylyl cyclase, epidermal growth factor receptor (EGFR), CD44, MUC5AC, Squamous cell carcinoma antigen, P62/c-myc (HGF receptor) and p53.

**Targets that are downregulated in Barrett's oesophagus:**
MUC1, glutathione S-transferase, retinoblastoma gene product.

A more preferred target that is downregulated in Barrett's oesophagus is MUC1.

**Targets that are overexpressed in squamous cell carcinoma of the oesophagus:**
Antigens and cell-surface receptors:
CD44, CD44v2, CD44v6, squamous cell carcinoma antigen (SCC), P62/c-myc (HGF receptor), c-erb2 (EGF receptor).

Proteins involved in cell cycle regulation or intracellular signalling:
MIB-1, p53, PCNA, survivin, CDC25A, CDC25B, cyclin D1, MDM2, p21.

Cytokeratins etc.:
CK 5/6, CK8, CK10,CK 13, CK18, CK19.

Others:
Endothelin, bFGF, proteins involved in angiogenesis, involucrin, cathepsin D, MMP-9.

More preferred targets that are overexpressed in squamous cell carcinoma of the oesophagus are: CD44, Squamous cell carcinoma antigen, matrix metalloproteinases, P62/c-myc (HGF receptor), p53 and EGFR/erB-2.

**Targets that are downregulated in squamous cell carcinoma of the oesophagus:**

Nm23-H1, E-cadherin, pRb, cyclin D1, axin, RCAS1, CK 20 and PAX9 gene product.

More preferred targets that are downregulated in squamous cell carcinoma of the oesophagus are E-cadherin and CK20.

**Targets that are overexpressed in adenocarcinoma and other carcinomas of the oesophagus:**

Antigens and cell-surface receptors:

ß-catenin, cholecystokinin receptors A and B, CD44V6, SCC, Tumor M2-PK, c-erb2, c-myc, , Guanylyl cyclase, integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, ligands of Helix pomatia lectin, MUC1, MUC4, Epidermal growth factor receptor (EGFR), c-erb2, c-met.

Enzymes:

COX-2, MMP-1, MMP-2, MMP-7, MMP-9, MMP-12, MMP-14, Cathepsin D, Pyrimidine nucleoside phosphorylase, telomerase.

Others:

TNF-α, CK7, involucrin, EF1 gamma, Mcm2, Mcm5, Ki-67, p53, TGF-α, EGF, FGF-1, c-src, c-ras.

More preferred targets that are overexpressed in adenocarcinoma and other carcinomas of the oesophagus are matrix metalloproteinases, CD44, COX-2, guanylyl cyclase, P62/c-myc (HGF receptor), p53 and EGFR/erB-2.

**Targets that are downregulated in adenocarcinoma and other carcinomas of the oesophagus:**

CK20, E-cadherin, Lamins A/C and B1, nm23.

More preferred targets that are downregulated in adenocarcinoma and other carcinomas of the oesophagus are E-cadherin and CK20.

Most preferred targets for both oesophageal cancer and Barrett's oesophagus are: E-cadherin, CD44, P62/c-myc (HGF receptor), p53 and EGFR/erB-2.

Generally, any targets that have been identified as possible targets for agents for treatment of oesophageal cancer and Barrett's oesophagus are potential targets also in optical imaging.

The preferred contrast agents of the present invention are molecules with relatively low molecular weights. The molecular weight of preferred contrast agents is below 14 000 Daltons, preferably below 10000 Daltons and more preferably below 7000 Daltons.

The contrast agents are preferably comprised of a vector that has affinity for an abnormally expressed target in oesophageal cancerous tissue, and an optical reporter.

Thus viewed from one aspect the present invention provides a contrast agent of formula I:

V-L-R (I)

wherein V is one or more vector moieties having affinity for one or more abnormally expressed target in oesophageal cancerous tissue, L is a linker moiety or a bond and R is one or more reporter moieties detectable in optical imaging.

The vector has the ability to direct the contrast agent to a region of oesophageal cancerous tissue. The vector has affinity for the abnormally expressed target and preferably binds to the target. The reporter is detectable in an optical imaging procedure and the linker must couple vector to reporter, at least until the reporter has been delivered to the region of oesophageal cancerous tissue and preferably until the imaging procedure has been completed.

The vector can generally be any type of molecule that has affinity for abnormally expressed target. The molecules should be physiologically acceptable and should preferably have an acceptable degree of stability. The vector is preferably selected from the following group of compounds: peptides, peptoids/peptidomimetics, oligonucleotides, oligosaccharides, lipid-related compounds like fatty-acids, traditional organic drug-like small molecules, synthetic or semi-synthetic, and derivatives and mimetics thereof. When the target is an enzyme the vector may comprise an inhibitor of the enzyme or an enzyme substrate. The vector of the contrast agent preferably has a molecular weight of less than 10 000 Daltons, more

preferably less than 4500 Daltons and most preferably less than 2500 Daltons, and hence does not include antibodies or internal image antibodies. In addition to problems with immune reactions, long circulation time and limited distribution volume, many antibodies have an affinity for the receptor that is too low for use in imaging.

An optical imaging contrast agent comprising a vector having affinity for any of the preferred targets is a preferred embodiment of the invention.

Contrast agents having affinity for more than one abnormally expressed target related to the disease is an aspect of the invention. Such contrast agents can comprise two or more different vectors or molecular subunits that target two or more different abnormally expressed targets.

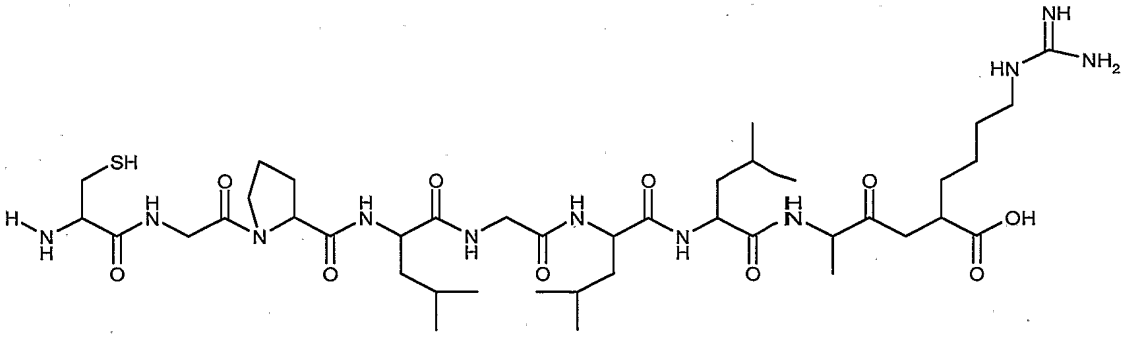
Another possibility according to the present invention is that the contrast agent comprises one vector that is able to bind to more than one abnormally expressed target in oesophageal cancer and Barrett's oesophagus.

A contrast agent according to the present invention can also comprise more than one vector of same chemical composition that bind to the abnormally expressed biological target.

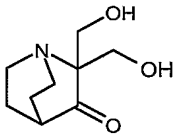
Some receptors are unique to endothelial cells and surrounding tissues. Examples of such receptors include growth factor receptors such as VEGF and adhesion receptors such as the integrin family of receptors. Peptides comprising the sequence arginine-glycine-aspartic acid (RGD) are known to bind to a range of integrin receptors. Such RGD-type peptides constitute one group of vectors for targets associated with oesophageal cancer and Barrett's oesophagus.

Below are some examples of vectors having affinity for oesophageal cancerous tissue-related abnormally expressed targets:

<u>Vectors for matrix metalloproteinases, such as for MMP-7:</u>
Peptide sequence: Cys-Gly-Pro-Leu-Gly-Leu-Leu-Ala-Arg-OH

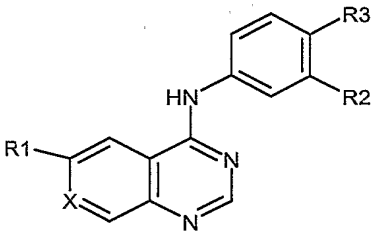


Vectors for p53:



A suggested synthesis is given in example 3.

Vectors for EGFR/erB-2:



Wherein

R1 = e.g. a substituted alkoxy, arylamide, and may include a chromophore.

R2 = halogen,

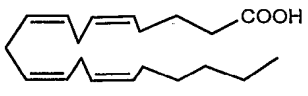
R3 = H, fluorine,

X = N or CR4, wherein R4 is alkoxy.

The vectors represent a group of tyrosine kinase inhibitors and are ATP analogues and analogues of the 4-anilinquinazoline skeleton.

Vectors for cyclo-oxygenase-2 (COX-2):

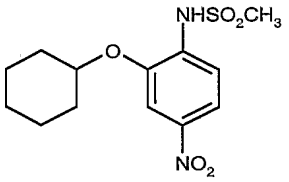
Arachidonic acid [506-32-1] (Sigma A9673, A8798):



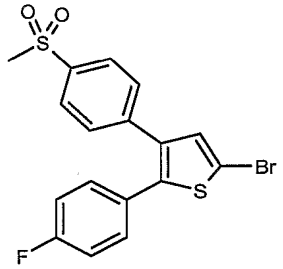

Arachidonic acid is the endogenous substrate for COX-2, and is an essential fatty acid and a precursor in the biosynthesis of prostaglandins.

Other vectors for COX-2 are exogenous compounds that bind to COX-2, for example so-called COX-2 inhibitors. The chemical classes of the main COX-2 inhibitors are shown in WO 02/07721.

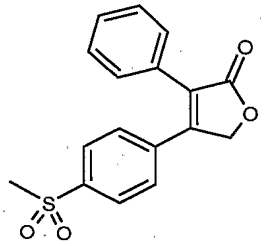
Such vectors include:



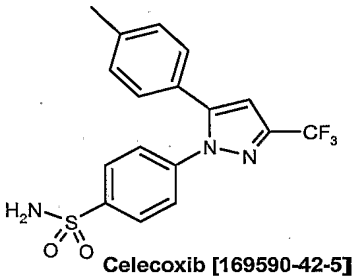

NS-398 (Sigma N-194)




DuP-697 (Sigma D4938)




Rofecoxib [162011-90-70]




Celecoxib [169590-42-5]

A wide variety of linkers can be used. The linker component of the contrast agent is at its simplest a bond between the vector and the reporter moieties. In this aspect the reporter part of the molecule is directly bound to the vector that binds to the abnormally expressed target. More generally, however, the linker will provide a mono- or multi-molecular skeleton covalently or non-covalently linking one or more vectors to one or more reporters, e.g. a linear, cyclic, branched or reticulate molecular skeleton, or a molecular aggregate, with in-built or pendant groups which bind covalently or non-covalently, e.g. coordinatively, with the vector and reporter moieties. The linker group can be relatively large in order to build into the contrast agent optimal size or optimal shape or simply to improve the binding characteristics

for the contrast agent to the abnormally expressed target in oesophageal cancerous tissue.

Thus, linking of a reporter unit to a desired vector may be achieved by covalent or non-covalent means, usually involving interaction with one or more functional groups located on the reporter and/or vector. Examples of chemically reactive functional groups which may be employed for this purpose include amino, hydroxyl, sulfhydroxyl, carboxyl and carbonyl groups, as well as carbohydrate groups, vicinal diols, thioethers, 2-aminoalcohols, 2-aminothiols, guanidinyl, imidazolyl and phenolic groups.

The reporter is any moiety capable of detection either directly or indirectly in an optical imaging procedure. The reporter might be a light scatterer (e.g. a coloured or uncoloured particle), a light absorber or a light emitter. More preferably the reporter is a dye such as a chromophore or a fluorescent compound. The dye part of the contrast agent can be any dye that interacts with light in the electromagnetic spectrum with wavelengths from the ultraviolet light to the near-infrared. Preferably, the contrast agent of the invention has fluorescent properties.

Preferred organic dye reporters include groups having an extensive delocalized electron system, eg. cyanines, merocyanines, indocyanines, phthalocyanines, naphthalocyanines, triphenylmethines, porphyrins, pyrilium dyes, thiapyrilium dyes, squarylium dyes, croconium dyes, azulenium dyes, indoanilines, benzophenoxazinium dyes, benzothiaphenothiazinium dyes, anthraquinones, napthoquinones, indathrenes, phthaloylacridones, trisphenoquinones, azo dyes, intramolecular and intermolecular charge-transfer dyes and dye complexes, tropones, tetrazines, bis(dithiolene) complexes, bis(benzene-dithiolate) complexes, iodoaniline dyes, bis(S,O-dithiolene) complexes. Fluorescent proteins, such as green fluorescent protein (GFP) and modifications of GFP that have different absorption/emission properties are also useful. Complexes of certain rare earth metals (e.g., europium, samarium, terbium or dysprosium) are used in certain contexts, as are fluorescent nanocrystals (quantum dots).

Particular examples of chromophores which may be used include fluorescein, sulforhodamine 101 (Texas Red), rhodamine B, rhodamine 6G, rhodamine 19, indocyanine green, Cy2, Cy3, Cy3B, Cy3.5, Cy5, Cy5.5, Cy7, Cy7.5, Marina Blue, Pacific Blue, Oregon Green 488, Oregon Green 514, tetramethylrhodamine, and

Alexa Fluor 350, Alexa Fluor 430, Alexa Fluor 532, Alexa Fluor 546, Alexa Fluor 555, Alexa Fluor 568, Alexa Fluor 594, Alexa Fluor 633, Alexa Fluor 647, Alexa Fluor 660, Alexa Fluor 680, Alexa Fluor 700, and Alexa Fluor 750. The cyanine dyes are particularly preferred.

Particularly preferred are dyes which have absorption maxima in the visible or near-infrared region, between 400 nm and 3 μm, particularly between 600 and 1300 nm.

The contrast agents according the invention can comprise more than one dye molecular sub-unit. These dye sub-units might be similar or different from a chemical point of view. Preferred contrast agents have less than 6 dye molecular sub-units.

Several relevant targets for oesophageal cancerous tissue are enzymes. A contrast agent for optical imaging of oesophageal cancerous tissue for targeting an enzyme can be an enzyme contrast agent substrate that can be transformed to a contrast agent product possessing different pharmacokinetic and/or pharmacodynamic properties from the contrast agent substrate. This embodiment of the invention provides contrast agent substrates having affinity for an abnormally expressed enzyme, wherein the contrast agent substrate changes pharmacodynamic and/or pharmacokinetic properties upon a chemical modification into a contrast agent product in a specific enzymatic transformation, and thereby enabling detection of areas of disease upon a deviation in the enzyme activity from the normal. Typical differences in pharmacodynamic and/or pharmacokinetic properties can be binding properties to specific tissue, membrane penetration properties, protein binding and solubility properties.

Alternatively, if the abnormally expressed target for diagnosis of oesophageal cancer and Barrett's oesophagus is an enzyme, the contrast agent for optical imaging can be a dye molecule that directly binds to the enzyme. The contrast agent will have affinity for the abnormally expressed enzyme, and this may be used to identify tissue or cells with increased enzymatic activity.

In a further aspect of the invention, the contrast agent changes dye characteristics as a result of an enzymatic transformation. For example, a fluorescent dye reporter of the contrast agent is quenched (no fluorescence) by associated quencher groups, until an enzymatic cleavage takes place, separating the dye from the quencher groups and resulting in fluorescence at the site of the abnormally expressed enzyme.

Another aspect of this part of the invention is that the dye may change colour, as e.g. a change in absorption and/or emission spectrum, as a result of an enzymatic transformation.

If the abnormally expressed target for diagnosis of oesophageal cancer and Barrett's oesophagus is a receptor or another non-catalytical target, the contrast agent for optical imaging can bind directly to the target and normally not change the dye characteristics.

The preferred contrast agents of the present invention are soluble in water. This means that the preferred contrast agents have a solubility in water at pH 7.4 of at least 1 mg/ml.

The contrast agents of the present invention can be identified by random screening, for example by testing of affinity for abnormally expressed targets of a library of dye labelled compounds either prepared and tested as single compounds or by preparation and testing of mixture of compounds (a combinatorial approach). Alternatively, random screening may be used to identify suitable vectors, before labelling with a reporter.

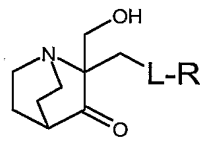
The contrast agents of the present invention can also be identified by use of technology within the field of intelligent drug design. One way to perform this is to use computer-based techniques (molecular modelling or other forms of computer-aided drug design) or use of knowledge about natural and exogenous ligands (vectors) for the abnormally expressed targets. The sources for exogenous ligands can for example be the chemical structures of therapeutic molecules for targeting the same target. One typical approach here will be to bind the dye chemical sub-unit (reporter) to the targeting vector so that the binding properties of the vector are not reduced. This can be performed by linking the dye at the far end away from the pharmacophore centre (the active targeting part of the molecule).

The contrast agents of the invention are preferably not endogenous substances alone. Some endogenous substances, for instance estrogen, have certain fluorescent properties in themselves, but they are not likely to be sufficient for use in optical imaging. Endogenous substances combined with an optical reporter however, fall within the contrast agents of the invention.

The contrast agents of the invention are intended for use in optical imaging. Any method that forms an image for diagnosis of disease, follow up of disease development or for follow up of disease treatment based on interaction with light in the electromagnetic spectrum from ultraviolet to near-infrared radiation falls within the term optical imaging. Optical imaging further includes all methods from direct visualization without use of any device and use of devices such as various scopes, catheters and optical imaging equipment, for example computer based hardware for tomographic presentations. The contrast agents will be useful with optical imaging modalities and measurement techniques including, but not limited to: luminescence imaging; endoscopy; fluorescence endoscopy; optical coherence tomography; transmittance imaging; time resolved transmittance imaging; confocal imaging; nonlinear microscopy; photoacoustic imaging; acousto-optical imaging; spectroscopy; reflectance spectroscopy; interferometry; coherence interferometry; diffuse optical tomography and fluorescence mediated diffuse optical tomography (continuous wave, time domain and frequency domain systems), and measurement of light scattering, absorption, polarisation, luminescence, fluorescence lifetime, quantum yield, and quenching.

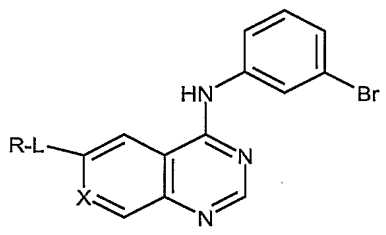
Some examples of contrast agents for optical imaging of oesophageal cancer and Barrett's oesophagus according to the invention are shown below:

Contrast agent with affinity for p53:




wherein L is a linker and R is a reporter according to the invention.

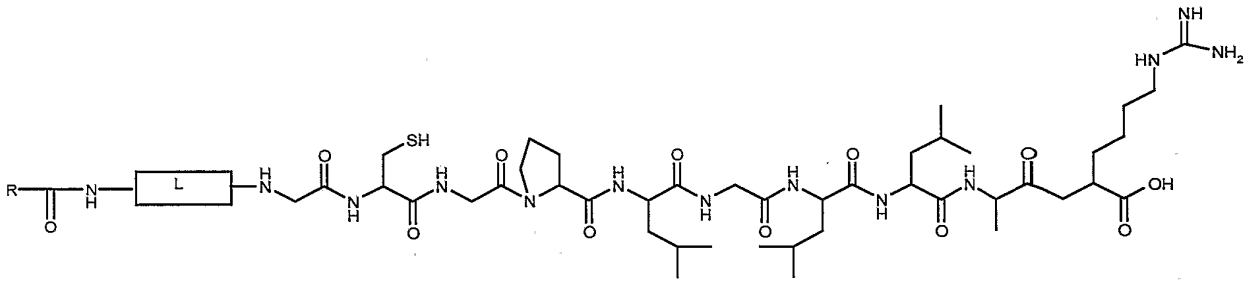
Contrast agent with affinity for EGFR/erB-2:



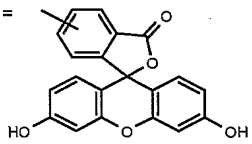
wherein X is nitrogen or CR', wherein R' is an alkoxy group, and wherein L is a linker and R a reporter according to the invention.

Contrast agent for mapping of matrix metalloproteinase

The peptide vector (Cys-Gly-Pro-Leu-Gly-Leu-Leu-Ala-Arg) is linked to e.g.fluorescein (R) through a linker (L):




R =




A synthesis for this is given in example 1.

A further embodiment is the use of contrast agents of the invention for optical imaging of oesophageal cancer and Barrett's oesophagus, that is for diagnosis of oesophageal cancer and Barrett's oesophagus, for use in follow up the progress in oesophageal cancer and Barrett's oesophagus development, for follow up the treatment of oesophageal cancer and Barrett's oesophagus, or for surgical guidance.

In the context of this invention, diagnosis includes screening of selected populations, early detection, biopsy guidance, characterisation, staging and grading. Follow up of treatment includes therapy efficacy monitoring and long-term follow-up of relapse. Surgical guidance includes tumour margin identification during resection.

Still another embodiment of the invention is a method of optical imaging of oesophageal cancer and Barrett's oesophagus using the contrast agents as described.

Still another embodiment of the invention is a method of optical imaging for diagnosis, to follow up the progress of oesophageal cancer and Barrett's oesophagus

development and to follow up the treatment of oesophageal cancer and Barrett's oesophagus, using a contrast agent as described.

One aspect of these methods is to administer the present contrast agents and follow the accumulation and elimination directly visually during surgery. Another aspect of these methods is to administer the present contrast agents and perform visual diagnosis through a gastroscope.

Still another aspect of the present invention is to administer the present contrast agents and perform the image diagnosis using computerized equipment as for example a tomograph.

Still another embodiment of the invention is use of a contrast agent as described for the manufacture of a diagnostic agent for use in a method of optical imaging of oesophageal cancer and Barrett's oesophagus involving administration of said diagnostic agent to an animate subject and generation of an image of at least part of said body, preferably the oesophagus or part of the oesophagus.

Still another embodiment of the invention is pharmaceutical compositions comprising one or more contrast agents as described or pharmaceutically acceptable salts thereof for optical imaging for diagnosis of oesophageal cancer and Barrett's oesophagus, for follow up progress of oesophageal cancer and Barrett's oesophagus development or for follow up the treatment of oesophageal cancer and Barrett's oesophagus. The contrast agents of the present invention may be formulated in conventional pharmaceutical or veterinary parenteral administration forms, e.g. suspensions, dispersions, etc., for example in an aqueous vehicle such as water for injections. Such compositions may further contain pharmaceutically acceptable diluents and excipients and formulation aids, for example stabilizers, antioxidants, osmolality adjusting agents, buffers, pH adjusting agents, etc. The most preferred formulation is a sterile solution for intravascular administration or for direct injection into area of interest. Where the agent is formulated in a ready-to-use form for parenteral administration, the carrier medium is preferably isotonic or somewhat hypertonic.

The dosage of the contrast agents of the invention will depend upon the clinical indication, choice of contrast agent and method of administration. In general,

however dosages will be between 1 micro gram and 70 grams and more preferably between 10 micro grams and 5 grams for an adult human.

While the present invention is particularly suitable for methods involving parenteral administration of the contrast agent, e.g. into the vasculature or directly into an organ or muscle tissue, intravenous administration being especially preferred, it is also applicable where administration is not via a parenteral route, e.g. where administration is transdermal, nasal, sub-lingual or is into an externally voiding body cavity, e.g. the gastrointestinal tract. The present invention is deemed to extend to cover such administration.

The following examples are illustrative only and not intended to be limiting. Other features and advantages of the invention will be apparent from the detailed description and from the claims.

**Examples:**

**Example 1. Contrast agent for mapping of matrix metalloproteinase (MMP). Synthesis of fluorescein–Cys-Gly-Pro-Leu-Gly-Leu-Leu-Ala-Arg-OH linker conjugate**

**Step 1**

The peptide component was synthesised on an ABI 433A automatic peptide synthesiser starting with Fmoc–Arg(Pmc)–wang resin on a 0.1 mmol scale using 1 mmol amino acid cartridges. The amino acids were pre-activated using HBTU before coupling. An aliquot of the peptide resin was then transferred to a clean round bottom flask an N-methyl morpholine (1 mmol) in DMF (5 ml) added followed by chloroacetyl chloride (1 mmol). The mixture was gently shaken until Kaiser test negative. The resin was extensively washed with DMF.

**Step 2**

5(6)–carboxyfluorescein (188 mg, 0.5 mmol) and dicyclohexylcarbodiimide (113 mg, 0.55 mmol) are dissolved in DMF (20 ml). The mixture is stirred for 2 hours and cooled to 0°C. A solution of hexamethylenediamide (116 mg, 1 mmol) and DMAP (30 mg) in DMF is added and the mixture is stirred at ambient temperature for 72 hours. The solution is evaporated and the conjugate between carboxyfluorescein and hexamethylene-amine is isolated as monoamide by chromatography (silica, chloroform and methanol).

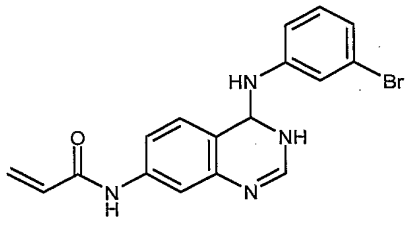
**Step 3**

The resin from step 1 is suspended in DMF (5 ml) and amide-amine conjugate from step 2 (0.5 mmol) pre-dissolved in DMF (5ml) containing triethylamine (0.5 mmol) is added. The mixture is heated to 50°C for 16 hours then excess reagents filtered off, following extensive washing with DMF, DCM and diethyl ether then air drying. The product is treated with TFA containing TIS (5%), $H_2O$ (5%), and phenol (2.5%) for 2 hours.

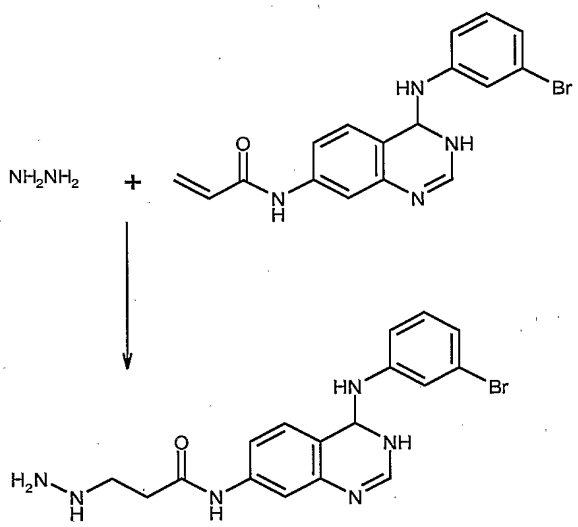
Excess TFA is removed *in vacuo* and the peptide is precipitated by the addition of diethyl ether. The crude peptide conjugate is purified by preparative HPLC C C-18, acetonitril, TFA, water).

**Example 2. Contrast agent for mapping of EGFR/erB-2 tyrosine kinase.**

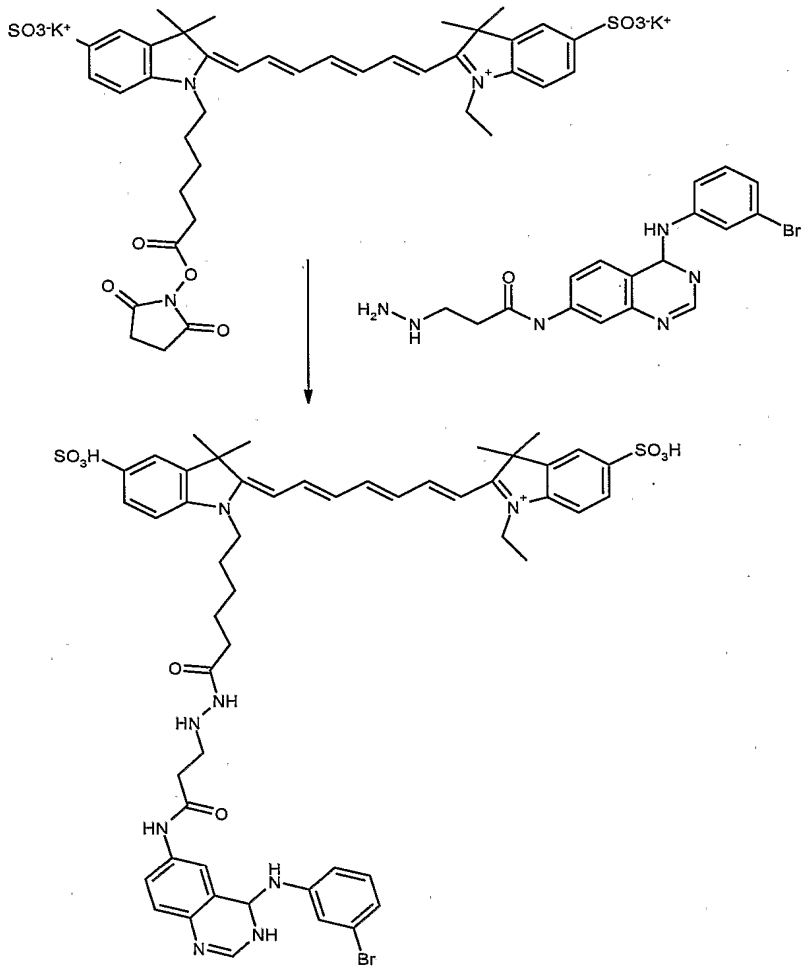
**Step 1.** N-[4-((3-bromophenyl)amino)quinazolin-7-y-] acrylamide is prepared according to J. B. Smaill et al in J. Med. Chem. (1999) 42 1803-1815.




**Step 2.** N-[4-((3-bromophenyl)amino)quinazolin-7-y-] acrylamide from step 1 (1 mmol) and ethylenediamine (10 mmol) are dissolved in DMF (25 ml). The mixture is stirred at 50 °C for 12 hours. The solvent is evaporated off and the conjugate compound is isolated by flash chromatography (silica, hexane, chloroform, methanol).



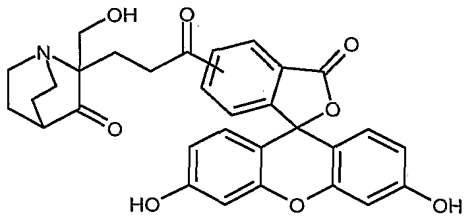

**Step 3.** Cy7-NHS ester (0.5 mmol), the conjugate compound from step 2 (0.5 mmol) and N-methylmorpholine (70 mg) are dissolved in DMF (30 ml). The mixture is stirred at 40 °C for 3 days. The Cy7 amide conjugate is isolated by flash chromatography (silica, hexane, ethyl acetate, methanol).




**Example 3. Contrast agent for binding to p53 oncoprotein (fluorescein)**

**Step 1.** Synthesis of 2,2-bis(hydroxymethyl)-1-aza-bicyclo[2,2,2,]octan-3-one. 3-quinuclidinone hydrochloride (Aldrich Q 190-5) (1 mmol) is dissolved in methanol-water (1:1, 30 ml). An aqueous solution of formaldehyde (37 %, 2.5 mmol) and sodium hydroxide (1.5 mmol) are added. The mixture is stirred for 12 hours at 50°C. The solvents are evaporated and the title compound isolated as free base using flash chromatography (silica, ethylacetate/chloroform, hexane).

**Step 2.**




5(6)-carboxyfluorescein (0.1 mmol) and dicyclohexyl carbodiimide (0.11 mmol) are dissolved in DMF. The mixture is stirred for 3 hours and cooled to 0 °C. A solution of 2,2-bis(hydrozymethyl)-1-azabicyclo[2,2,2] octane-3-one (0.5 mmol) and DMAP (10 mg) in DMF is added and the mixture is stirred at ambient temperature for 72 hours. The solution is evaporated and the contrast agent is isolate by flash chromatography (silica, ethyl acetate/hexane).

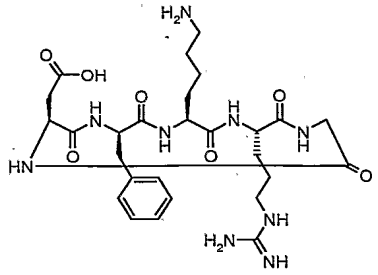
**Example 4. Contrast agent with affinity for integrins: RGD peptide linked to Cy5.5**

**Step 1. Assembly of amino acids**

The peptide sequence Asp-D-Phe-Lys-Arg-Gly was assembled on an Applied Biosystems 433A peptide synthesizer starting with 0.25 mmol Fmoc-Gly-SASRIN resin. An excess of 1 mmol pre-activated amino acids (using HBTU; O-Benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosohate) was applied in the coupling steps. The cleavage of the fully protected peptide from the resins was carried out by treatment of the resin with three portions of 35 mL of 1 % trifluoroacetic acid (TFA) in dichloromethane (DCM) for 5 minutes each. The filtrates containing the peptide was immediately neutralised with 2 % piperidine in DCM. The organics were extracted with water (3 x 100 mL), dried with $MgSO_4$ and evaporated *in vacuo*. Diethyl ether was added to the residue and the precipitate washed with ether and air-dried affording 30 mg of crude protected peptide. The product was analysed by analytical HPLC (conditions: Gradient, 20-70 % B over 10 min where A = $H_2O$/0.1 % TFA and B = $CH_3CN$/0.1 % TFA; flow, 2 mL/min; column, Phenomenex Luna 3$\mu$ 5 x 4.6 mm; detection, UV 214 nm; product retention time 7.58 min). Further product characterisation was carried out using electrospray mass spectrometry ($MH^+$ calculated, 1044.5; $MH^+$ found, 1044.4).

**Step 2. N-C Cyclisation**

c[-Asp-D-Phe-Lys-Arg-Gly-]

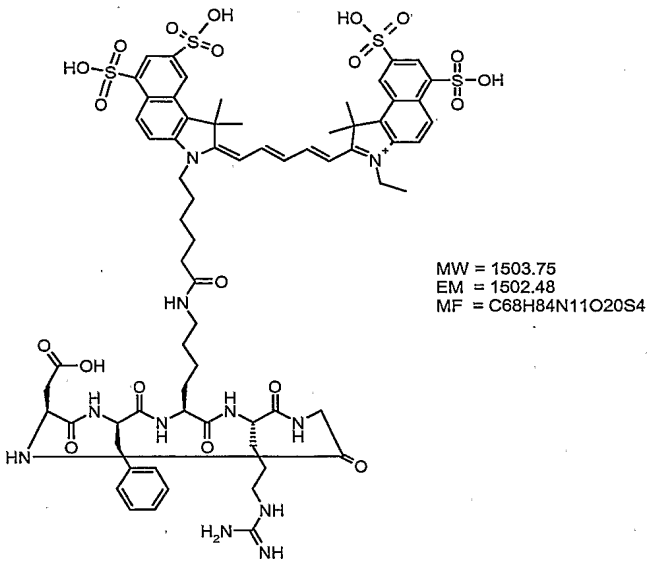


MW = 603.68
EM = 603.31
MF = C27H41N9O7

30 mg of the fully protected peptide, 16 mg of PyAOP, 4 mg of HOAt and 6 $\mu$L of N-methylmorpholine (NMM) were dissolved in dimethylformamide/DCM (1:1) and stirred over night. The mixture was evaporated *in vacuo* and diethyl ether added to the residue. The precipitate was washed with ether and air-dried. The crude cyclic fully protected peptide was treated with a solution of 25 mL TFA containing 5 % water, 5 % triisopropylsilane and 2.5 % phenol for two hours. TFA was evaporated *in vacuo* and diethyl ether added to the residue. The precipitate was washed with ether and air-dried. Purification by preparative RP-HPLC (0-30 % B over 40 min, where A = $H_2O$/0.1 % TFA and B = $CH_3CN$/0.1 % TFA, at a flow rate of 10 mL/min on a Phenomenex Luna 5 $\mu$ C18 250 x 21.20 mm column) of the crude material afforded 2.3 mg pure product peptide.The pure product was analysed by analytical HPLC (conditions: Gradient, 0-15 % B over 10 min where A = $H_2O$/0.1 % TFA and B = $CH_3CN$/0.1 % TFA; flow, 2 mL/min; column, Phenomenex Luna 3$\mu$ 5 x 4.6 mm; detection, UV 214 nm; product retention time 6.97 min). Further product characterisation was carried out using electrospray mass spectrometry ($MH^+$ calculated, 604.3; $MH^+$ found, 604.4).

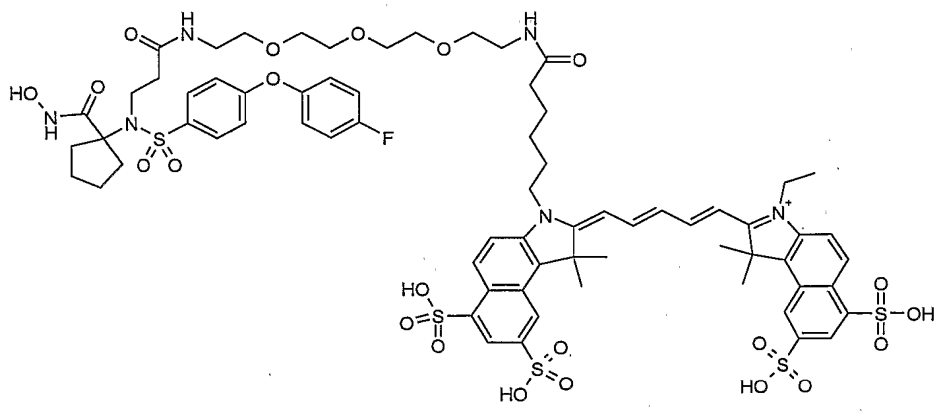
**Step 3 . Conjugation of Cy5.5 to RGD peptide**

c[-Asp-D-Phe-Lys(Cy5.5)-Arg-Gly-]




0.6 mg of the RGD peptide, 1.7 mg of Cy5.5 mono NHS ester and 5 $\mu$L of NMM were dissolved in 1 mL of dimethylformamide (DMF) and the reaction mixture stirred for 2 hrs. Diethyl ether was added to the DMF solution and the blue precipitate washed with diethyl ether and air-dried affording 0.7 mg of crude RGD peptide conjugated to Cy5.5.The pure product was analysed by analytical HPLC (conditions: Gradient, 5-50 % B over 10 min where A = $H_2O$/0.1 % TFA and B = $CH_3CN$/0.1 % TFA; flow, 0.3 mL/min; column, Phenomenex Luna 3$\mu$ 5 x 2 mm; detection, UV 214 nm; product retention time 8.32 min). Further product characterisation was carried out using electrospray mass spectrometry ($MH^+$ calculated, 1502.5; $MH^+$ found, 1502.6).

**Example 5. Synthesis of 3-[(4'-Fluorobiphenyl-4-sulfonyl)-(1-hydroxycarbamoylcyclopentyl)amino]propionic acid (compound A) derivatised with Cy5.5 – contrast agent for binding to MMP**



**a) 1,11-Diazido-3,6,9-trioxaundecane**

A solution of dry tetraethylene glycol (19.4 g, 0.100 mol) and methanesulphonyl chloride (25.2 g, 0.220 mol) in dry THF (100 ml) was kept under argon and cooled to 0 °C in an ice/water bath. To the flask was added a solution of triethylamine (22.6 g, 0.220 mol) in dry THF (25 ml) dropwise over 45 min. After 1 hr the cooling bath was removed and stirring was continued for 4 hrs. Water (60 ml) was added. To the mixture was added sodium hydrogencarbonate (6 g, to pH 8) and sodium azide (14.3 g, 0.220 mmol), in that order. THF was removed by distillation and the aqueous solution was refluxed for 24 h (two layers formed). The mixture was cooled and ether (100 ml) was added. The aqueous phase was saturated with sodium chloride. The phases were separated and the aqueous phase was extracted with ether (4 x 50 ml). Combined organic phases were washed with brine (2 x 50 ml) and dried ($MgSO_4$). Filtration and concentration gave 22.1 g (91%) of yellow oil. The product was used in the next step without further purification.

**b) 11-Azido-3,6,9-trioxaundecanamine**

To a mechanically, vigorously stirred suspension of 1,11-diazido-3,6,9-trioxaundecane (20.8 g, 0.085 mol) in 5% hydrochloric acid (200 ml) was added a solution of triphenylphosphine (19.9 g, 0.073 mol) in ether (150 ml) over 3 hrs at room temperature. The reaction mixture was stirred for additional 24 hrs. The phases were separated and the aqueous phase was extracted with dichloromethane (3 x 40 ml). The aqueous phase was cooled in an ice/water bath and pH was adjusted to ca 12 by addition of KOH. The product was extracted into dichloromethane (5 x 50 ml). Combined organic phases were dried ($MgSO_4$). Filtration and evaporation gave 14.0 g (88%) of yellow oil. Analysis by MALDI-TOF mass spectroscopy (matrix: □-cyano-4-hydroxycinnamic acid) gave a M+H peak at 219 as expected. Further characterisation using $^{1}H$ (500 MHz) and $^{13}C$ (125 MHz) NMR spectroscopy verified the structure.

**c) Linking compound A to PEG(4)-$N_3$**

To a solution of compound A (CP-471358, Pfizer, 41 mg, 87 μmol) in DMF (5 ml) were added 11-azido-3,6,9-trioxaundecanamine (19 mg, 87 μmol), HATU (Applied Biosystems, 33 mg, 87 μmol) and DIEA (Fluka, 30 μl, 174 μmol). After one hour reaction time the mixture was concentrated and the residue was purified by preparative HPLC (column Phenomenex Luna C18(2) 5 μm 21.2 x 250 mm, solvents: A = water/0.1% TFA and B = acetonitrile/0.1% TFA; gradient 30-60% B over 60 min; flow 10.0 ml/min, UV detection at 214 nm), giving 33.9 mg (59%) of product after

lyophilisation. LC-MS analysis (column Phenomenex Luna C18(2) 3 μm 50 x 4.60 mm, solvents: A = water/0.1% TFA and B = acetonitrile/0.1% TFA; gradient 20-100% B over 10 min; flow 1 ml/min, UV detection at 214 nm, ESI-MS) gave a peak at 4.88 min with m/z 667.4 ($MH^+$) as expected.

**d) Synthesis of compound A-PEG(4)-$NH_2$**

To a solution of the PEG(4)-$N_3$ compound from c) (4.7 mg, 7.0 μmol) in methanol (4 ml) was added Pd/C (Koch-Light, ca 10 mg) added. The mixture was stirred at room temperature under hydrogen atmosphere (1 atm) for 10 min. The mixture was filtered and concentrated. LC-MS analysis (column Phenomenex Luna C18(2) 3 μm 50 x 4.60 mm, solvents: A = water/0.1% TFA and B = acetonitrile/0.1% TFA; gradient 20-100% B over 10 min; flow 1 ml/min, UV detection at 214 nm, ESI-MS) gave a peak at 4.17 min with m/z 641.4 ($MH^+$) as expected. The product was used directly in the next step without further purification.

**e) Conjugation of Cy 5.5**

To a solution of the amine from d) (1.0 mg, 1.5 μmol) in DMF (0.2 ml) was added Cy 5.5-NHS (Amersham Biosciences, 1.0 mg, 1.0 μmol) and N-methylmorpholine (1 μl, 9 μmol). The reaction mixture was stirred for 48 h. MS analysis of the solution gave a spectrum showing starting material and the conjugated product at m/z 1539.7 ($M^{+)}$, expected 1539.4.

**Example 6: Cy5-TIMP-1**

Five micrograms of tissue inhibitor of metalloproteinases-1 (TIMP-1, cat.no. 970-TM) (carrier-free, from R&D Systems) were dissolved in 25 μl of 0.02 M borate buffer, pH 8.5. To this solution was added 2.5 nmol of the N-hydroxysuccinimide ester of a carboxylic acid derivative of Cy5 (Amersham Biosciences), dissolved in 5 μl of the same buffer. The reaction mixture was incubated for one hour in the dark at room temperature. Unreacted dye was separated from the fluorescent protein derivative by centrifuging through a Micro-Spin 6 gel filtration column (Bio-Rad, exclusion limit about 6 kDa). The eluate fluoresced with excitation light at 646 nm, the emission being measured at 678 nm. The product was a fluorescent targeting molecule for matrix metalloproteinases.

**Example 7: Fluorescein-TIMP-1**

Five micrograms of tissue inhibitor of metalloproteinases-1 (TIMP-1, cat.no. 970-TM) (carrier-free, from R&D Systems) were dissolved in 25 μl of 0.02 M borate buffer, pH

8.5. To this solution was added 2.5 nmol of the N-hydroxysuccinimide ester of a carboxylic acid derivative of fluorescein (Fluka), dissolved in 5 µl of the same buffer. The reaction mixture was incubated for one hour in the dark at room temperature. Unreacted dye was separated from the fluorescent protein derivative by centrifuging through a Micro-Spin 6 gel filtration column (Bio-Rad, exclusion limit about 6 kDa). The eluate fluoresced with excitation light at 485 nm, the emission being measured at 538 nm. The product was a fluorescent targeting molecule for matrix metalloproteinases.

**Example 8: Cy5-EGF**

Sixty micrograms of epidermal growth factor (EGF, cat.no. 236-EG, 10 nmol) (from R&D Systems) were dissolved in 10 µl of 0.02 M borate buffer, pH 8.5. To this solution was added 10 µl buffer and 50 nmol of the N-hydroxysuccinimide ester of a carboxylic acid derivative of Cy5 (Amersham Biosciences). The reactive dye was dissolved in 5 µl of the same buffer, mixed 1:1 with dioxan. The reaction mixture was incubated for one hour in the dark at room temperature. Unreacted dye was separated from the fluorescent protein derivative by centrifuging through a Micro-Spin 6 gel filtration column (Bio-Rad, exclusion limit about 6 kDa). The eluate, which was bright blue, fluoresced with excitation light at 646 nm, the emission being measured at 678 nm. The product was a fluorescent targeting molecule for the epidermal growth factor receptor.

**Example 9: Cy7.5-EGF**

Sixty micrograms of epidermal growth factor (EGF, cat.no. 236-EG, 10 nmol) (from R&D Systems) were dissolved in 10 µl of 0.02 M borate buffer, pH 8.5. To this solution was added 10 µl buffer and 50 nmol of the N-hydroxysuccinimide ester of a carboxylic acid derivative of Cy7.5 (Amersham Biosciences). The reactive dye was dissolved in 5 µl of the same buffer, mixed 1:1 with dioxan. The reaction mixture was incubated for one hour in the dark at room temperature. Unreacted dye was separated from the fluorescent protein derivative by centrifuging through a Micro-Spin 6 gel filtration column (Bio-Rad, exclusion limit about 6 kDa). The eluate, which was dark green, fluoresced with excitation light at 700 nm, the emission being measured at 790 nm. The product was a fluorescent targeting molecule for the epidermal growth factor receptor.

**Example 10: Fluorescein-EGF**

Sixty micrograms of epidermal growth factor (EGF, cat.no. 236-EG , 10 nmol) (from R&D Systems) were dissolved in 10 $\mu$l of 0.02 M borate buffer, pH 8.5. To this solution was added 10 $\mu$l buffer and 50 nmol of the N-hydroxysuccinimide ester of a carboxylic acid derivative of fluorescein (Fluka), dissolved in 5 $\mu$l of dioxan. The reaction mixture was incubated for one hour in the dark at room temperature. Unreacted dye was separated from the fluorescent protein derivative by centrifuging through a Micro-Spin 6 gel filtration column (Bio-Rad, exclusion limit about 6 kDa). The eluate, which was yellow, fluoresced with excitation light at 485 nm, the emission being measured at 538 nm. The product was a fluorescent targeting molecule for the epidermal growth factor receptor.

**Claims:**

1. An optical imaging contrast agent with affinity for an abnormally expressed biological target associated with oesophageal cancer or Barrett's oesophagus.

2. A contrast agent as claimed in claim 1 with molecular weight below 14 000 Daltons.

3. A contrast agent as claimed in claim 1 or 2 of formula I

V-L-R, (I)

wherein V is one or more vector moieties having affinity for an abnormally expressed target in oesophageal cancer or Barrett's oesophagus, L is a linker moiety or a bond and R is one ore more reporter moieties detectable in optical imaging.

4. A contrast agent as claimed in any of claims 1 to 3 comprising a contrast agent substrate, wherein the target is an abnormally expressed enzyme, such that the contrast agent changes pharmacodynamic properties and/or pharmacokinetic properties upon a chemical modification from a contrast agent substrate to a contrast agent product upon a specific enzymatic transformation.

5. A contrast agent as claimed in any of claims 1 to 4 having affinity for any of the targets selected from E-cadherin, CD44, P62/c-myc (HGF receptor), p53 and EGFR/erB-2.

6. A contrast agent as claimed in any of claims 3 to 5 wherein V is selected from peptides, peptoid moieties, oligonucleotides, oligosaccharides, fat-related compounds and traditional organic drug-like small molecules.

7. A contrast agent as claimed in any of claims 3-6 wherein R is a dye that interacts with light in the wavelength region from the ultraviolet to the near-infrared part of the electromagnetic spectrum.

8. A pharmaceutical composition for optical imaging of oesophageal cancer and Barrett's oesophagus comprising a contrast agent as defined in any of claims 1 to 7 together with at least one pharmaceutically acceptable carrier or excipient.

9. Use of a contrast agent as claimed in any of claims 1 to 7 for the manufacture of a diagnostic agent for use in a method of optical imaging of oesophageal cancer and Barrett's oesophagus involving administration of said diagnostic agent to an animate subject and generation of an image of at least part of said subject.

10. A method of optical imaging of oesophageal cancer and Barrett's oesophagus of an animate subject involving administering a contrast agent as defined in any of claims 1 to 7to the subject and generating an optical image of at least a part of said subject to which said contrast agent has distributed.

11. Method as claimed in claim 10 for diagnosis of oesophageal cancer and Barrett's oesophagus, for follow up of the progress of oesophageal cancer and Barrett's oesophagus development, follow up of treatment of oesophageal cancer and Barrett's oesophagus, or in surgical guidance.

12. Use of a contrast agent as defined in any of claims 1 to 7 for optical imaging of oesophageal cancer and Barrett's oesophagus.

13. Use of a contrast agents as claimed in claim 12 for diagnosis of oesophageal cancer and Barrett's oesophagus, for follow up of the progress of oesophageal cancer and Barrett's oesophagus development, for follow up of treatment of oesophageal cancer and Barrett's oesophagus, or in surgical guidance.

SEQUENCE LISTING

<110> Amersham Health AS

<120> Optical imaging contrast agents

<130> PN0397

<140> NO20035682

<141> 2003-12-18

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<170> PatentIn version 3.1

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# INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/NO2004/000393

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 7 A61K49/00

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

*Minimum documentation searched (classification system followed by classification symbols)*
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, EMBASE, CHEM ABS Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | EP 0 800 831 A (DAIICHI PURE CHEMICALS CO. LTD; DAIICHI PURE CHEMICALS CO., LTD)<br>15 October 1997 (1997-10-15)<br>page 2, line 50<br>page 7, line 37<br>example 12<br>page 17, lines 27-49<br>claim 1<br>-----<br>-/-- | 1,3,5,<br>7-13 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° *Special categories of cited documents :*

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international *filing date*

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or *other means*

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, *such combination being obvious to a person skilled* in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 9 May 2005 | 23/05/2005 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| *European Patent Office*, P.B. 5818 Patentlaan 2<br>NL – 2280 HV Rijswijk<br>Tel. (+31–70) 340–2040, Tx. 31 651 epo nl,<br>Fax: (+31–70) 340–3016 | Veronese, A |

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
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**Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 10-13 are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

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